THE EFFICACY OF PROTOPORPHYRIN AS A PREDICTIVE BIOMARKER FOR LEAD EXPOSURE IN CANVASBACK DUCKS: EFFECT OF SAMPLE STORAGE TIME*

J. CHRISTIAN FRANSON[1], WILLIAM L. HOHMAN[2], JOSEPH L. MOORE[2]** and MILTON R. SMITH[1]

[1] *National Biological Service, National Wildlife Health Center, 6006 Schroeder Road, Madison, Wisconsin 53711, U.S.A.;* [2] *National Biological Service, Southern Science Center, 700 Cajundome Boulevard, Lafayette, Louisiana 70506, U.S.A.*

(Received: October 1995; revised: January 1996)

Abstract. We used 363 blood samples collected from wild canvasback ducks (*Aythya valisineria*) at Catahoula Lake, Louisiana, U.S.A. to evaluate the effect of sample storage time on the efficacy of erythrocytic protoporphyrin as an indicator of lead exposure. The protoporphyrin concentration of each sample was determined by hematofluorometry within 5 min of blood collection and after refrigeration at 4 °C for 24 and 48 h. All samples were analyzed for lead by atomic absorption spectrophotometry. Based on a blood lead concentration of ≥ 0.2 ppm wet weight as positive evidence for lead exposure, the protoporphyrin technique resulted in overall error rates of 29%, 20%, and 19% and false negative error rates of 47%, 29% and 25% when hematofluorometric determinations were made on blood at 5 min, 24 h, and 48 h, respectively. False positive error rates were less than 10% for all three measurement times. The accuracy of the 24-h erythrocytic protoporphyrin classification of blood samples as positive or negative for lead exposure was significantly greater than the 5-min classification, but no improvement in accuracy was gained when samples were tested at 48 h. The false negative errors were probably due, at least in part, to the lag time between lead exposure and the increase of blood protoporphyrin concentrations. False negatives resulted in an underestimation of the true number of canvasbacks exposed to lead, indicating that hematofluorometry provides a conservative estimate of lead exposure.

1. Introduction

Ingested lead shot has long been recognized as a source of lead poisoning in waterfowl (Bellrose, 1959; Sanderson and Bellrose, 1986) and has also been reported in a variety of other avian species (Locke and Friend, 1992). Nontoxic shot requirements for waterfowl hunting were implemented throughout the United States in 1991 (Anderson, 1992). However, ingestion of lead shot remains a problem at some sites where shot densities are high and characteristics of wetland sediments impede settlement or burial of shot (Hohman et al., 1995; Mauser et al., 1990; Sanderson and bellrose, 1986; U.S. Department of Interior, 1986). Poisoning by lead from other sources, including fishing weights (Pokras and Chafel, 1992; Birkhead, 1982; Locke et al., 1982), bullet fragments (MacDonald et al., 1983), paint chips (Sileo and Fefer, 1987), and mining wastes (Blus et al., 1991; Chupp and Dalke, 1964)

* The U.S. Government's right to retain a non-exclusive, royalty-free licence in and to any copyright is acknowledged.
** Deceased.

Environmental Monitoring and Assessment **43:** 181–188, 1996.

has also been reported in waterfowl and other avian species. Lead exposure from atmospheric or other environmental contamination occurs in birds near smelters and highways (Beyer et al., 1985; Henny et al., 1994; Grue et al., 1986), in cities (Hutton, 1980), and in industrialized areas (Bull et al., 1983).

The persistence of lead as a hazard to wildlife in a variety of situations results in the continued need of techniques for monitoring exposure and assessing the magnitude of injury. Of the nondestructive techniques available for evaluating lead exposure in avian populations, including analysis of blood for lead and delta-aminolevulinic dehydratase (ALAD) activity (Dieter et al., 1976; Friend, 1985; Hoffman et al., 1985; Beyer et al., 1988), the quantitative determination of erythrocytic protoporphyrin (EPP) by hematofluorometry (Roscoe et al., 1979) has several advantages. Minimal equipment and supplies are needed, testing can be done under field conditions with an electrical power source, only a small blood sample is required, and the assay cost is quite low.

Roscoe et al. (1979) found that EPP concentrations were rarely elevated in blood freshly drawn from lead-poisoned mallard ducks (*Anas platyrhynchos*). However, when blood samples were oxygenated and refrigerated the protoporphyrin concentrations increased until the second day and then remained relatively constant for at least seven more days (Roscoe et al., 1979). This was attributed to *in vitro* synthesis of protoporphyrin and the authors recommended that blood samples be refrigerated for 48 h before testing. Refrigerated storage of blood for 48 h is often inconvenient or impossible in field situations, particularly if live birds must be held while lead exposure is evaluated. If storage time could be reduced, the EPP technique would be logistically simpler and more easily applied.

Sediments of Catahoula Lake, Louisiana, contain high densities of spent lead shot (Wills and Glasgow, 1964) and about 27% of canvasbacks (*Aythya valisineria*) collected there during the winter of 1987–1988 had lead shot in their gizzards (Hohman et al., 1990), providing a field situation with a ready source of lead-exposed birds. In conjunction with a study of winter survival of immature canvasbacks at Catahoula Lake (Hohman et al., 1995), we evaluated the effect of blood sample storage time on the efficacy of EPP determination for predicting lead exposure.

2. Methods

Canvasbacks wintering at Catahoula Lake in 1991–92, 1992–93, and 1993–94 were captured for the purpose of implanting radio transmitters as described by Hohman et al. (1995). Blood samples of 2 ml each were collected from 363 birds by jugular venipuncture into sodium heparinized evacuated glass tubes (Vacutainer®, Becton-Dickinson and Company. Rutherford, New Jersey) that were immediately placed on a rotating mixer for about 5 min. The stopper was removed from the Vacutainer® and a drop of blood was collected in a glass capillary tube and delivered to a glass coverslip for EPP determination with a hematofluormeter (AVIV® Biomedical, Incorporated, Lakewood, New Jersey) modified according to Roscoe et al. (1979). After this initial EPP measurement, the stoppers were replaced and Vacutainers® were refrigerated at 4 °C. Two additional EPP measurements were taken as above, at 24 h (± 15 min) and 48 h (± 15 min) after the initial reading. After the last EPP measurements, blood samples were frozen at –18 °C until analyzed for lead by graphite furnace atomic absorption spectrophotometry as described by DeStefano et al. (1991). The lower limit of detectable lead residue was 0.02 ppm wet weight, and the average recovery of lead from six control samples of known concentrations was 99.8%. An estimate of analytical precision (Taylor, 1987) for this method was made using duplicate analysis of 123 mallard blood samples; 71 had lead concentrations within the range of 0.04 to 0.2 ppm wet weight, and 52 had lead concentrations $\geq$ 0.2 ppm. The 95% confidence intervals for blood lead concentrations of 0.04 to 0.2 ppm and $\geq$ 0.2 ppm were ± 12.0% and ± 6.1%, respectively.

Blood samples with lead concentrations of $\geq$ 0.2 ppm wet weight were classified as possitive for lead exposure. Dieter et al. (1976) reported that 0.2 ppm lead in the blood of canvasbacks resulted in a 75% inhibition of ALAD activity and suggested that this level was the lower threshold indicative of harmful lead exposure in this species. Samples with < 0.2 ppm lead were considered evidence of normal background exposure. Knowing the blood lead concentrations, we sought to determine the EPP concentrations indicative of the positive and negative populations. We compared blood lead results with the 5-min EPP reading and, using discriminant analysis (SAS/STAT User's Guide, 1990), determined the EPP concentration that best separated the positive and negative populations. This was repeated for the 24 h and 48 h EPP measurements. The positive EPP population was additive in that all EPP values that fell within the positive range at a given time period were automatically classified positive at each later time period. Samples with EPP values in the negative range, however, were re-examined at each later time period. For example, the EPP concentration of a blood sample that was classified within the negative range at the 5-min measurement was re-examined at the 24 h measurement to determine if its value had increased enough to then fall in the positive range. If it was positive at 24 h it was considered positive at 48 h; if it was negative at 24 h, it was evaluated again at 48 h. We used McNemar's test (Zar, 1984) to compare the accuracy of the EPP classifications as the sample holding time increased from 5 min to 24 and 48 h.

3. Results

Of the 363 blood samples, 216 (60%) had lead concentrations $\geq$ 0.2 ppm wet weight and were classified as positive for lead exposure (Figure 1). At all three measurement times, mean EPP concentrations were several times higher for blood samples

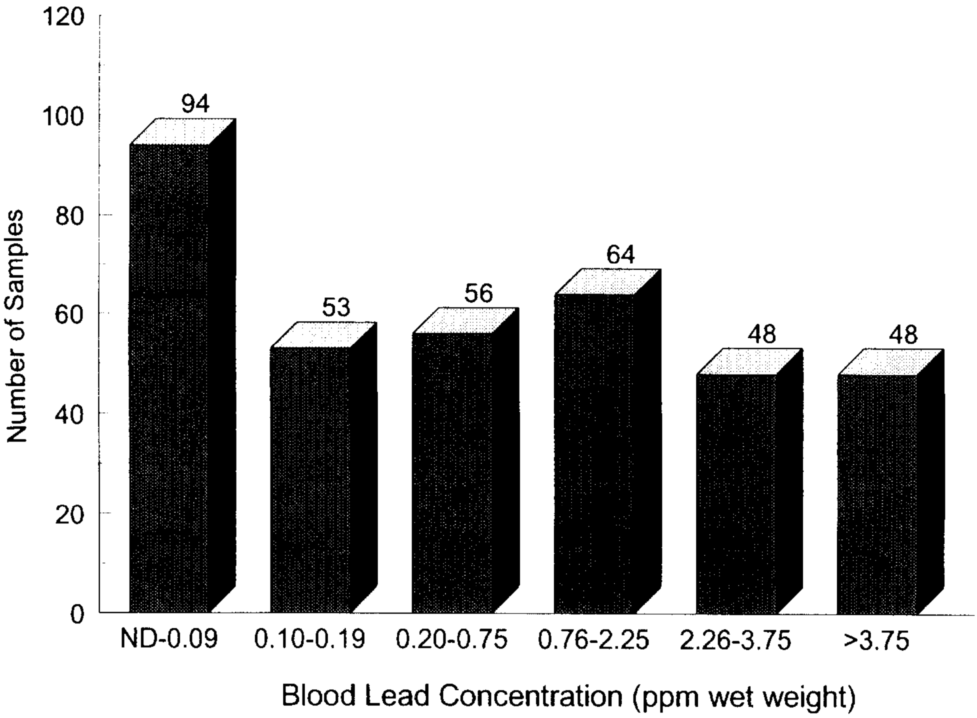

Figure 1. Distribution of lead concentrations in 363 blood samples from canvasbacks at Catahoula Lake, Louisiana.

Table I

Mean (± SD [range]) protoporphyrin concentrations (μg/dl) of blood samples collected from canvasbacks with lead concentrations $\geq$ 0.2 ppm (positive) and $<$ 0.2 ppm (negative) read within 5 min (initial) of blood collection and after refrigeration at 4 °C for 24 and 48 h.

Lead exposure	N	Holding time: Initial	24 h	48 h
Positive	216	25 ± 29 (4–134)	78 ± 59 (7–172)	83 ± 58 (7–174)
Negative	147	7 ± 2 (5–20)	15 ± 7 (7–81)	17 ± 8 (8–83)

that were positive for lead exposure than for those classified as negative (Table I). However, the EPP concentrations and variation increased after refrigeration in both the lead-exposed and nonexposed groups, particularly between the 5-min and 24 h readings (Table I). The EPP concentrations, obtained by discriminant classification criteria, that best separated the positive and negative blood lead populations were 10.5 μg/dl EPP at 5 min 24.5 μg/dl at 24 h, and 27.5 μg/dl at 48 h (Table II). The use of these EPP values to predict lead exposure resulted in false positive classification rates of 3%, 7%, and 9% and false negative classification rates of 47%,

Table II

Canvasback blood lead concentrations in comparison to blood protoporphyrin concentrations determined within 5 min of blood collection (initial) and after refrigeration at 4 °C for 24 and 48 h.

Blood lead[b]	Blood Protoporphyrin[a] Initial ≥ 10.5	Initial < 10.5	Initial Error[c]	24 h ≥ 24.5	24 h < 24.5	24 h Error	48 h ≥ 27.5	48 h < 27.5	48 h Error
≥ 0.2	114	102	47	154	62	29	161	55	25
< 0.2	5	142	3	11	136	7	13	134	9

[a] μg/dl.
[b] ppm wet weight: ≥ 0.2 = positive for lead exposure; < 0.2 = negative.
[c] % samples incorrectly classified by hematofluorometry.

Table III

Comparison of true rates of lead exposure in canvasbacks (blood lead ≥ 0.2 ppm, wet weight) with rates estimated by initial (< 5 min after blood collection), 24 h, and 48 h protoporphyrin determinations

	Protoporphyrin classification Initial	Initial + 24 h	Initial + 24 h + 48 h
No. correct	256	290[a]	295[b]
No. incorrect	107	73	68
% error	29	20	19

[a] McNemar's test indicates significant difference from initial value ($Z = 5.31$, $P < 0.001$).
[b] McNemar's test indicates no difference from initial + 24 h value ($Z = 1.67$, $P = 0.10$).

29%, and 25% at the 5-min, 24 h, and 48 h readings, respectively (Table II). The accuracy of the 24 h EPP classification of blood samples as positive or negative for lead exposure was significantly greater than the initial 5-min classification, but no improvement in accuracy was gained when samples were held for 48 h (Table III). The overall rates of error (false positive + false negatives/363) for the 5-min, 24 h, and 48 h EPP measurements were 29%, 20%, and 19%, respectively (Table III).

4. Discussion

In human medicine, hematofluorometric determination of zinc protoporphyrin is a well-establishd screening technique for lead exposure (Piomelli, 1987; Lamola et al., 1975; Mushak, 1992). Screening for lead exposure in birds is based on similar principles, although modifications to the hematofluorometer are required because metal-free protoporphyrin is measured and current methods recommend refrigeration of samples for 48 h to allow for *in vitro* synthesis of EPP (Roscoe et al., 1979). This technique for EPP determination has been used in a variety of avian species to evaluate lead exposure in field and experimental studies, often in conjunction with other indicators (Henny et al., 1994; Blus et al., 1991; Beyer et al., 1988; Franson et al., 1986; Roscoe et al., 1979).

Using data collected from lead-dosed mallards, Roscoe et al. (1979) recommended that blood samples for hematofluorometry be held for 48 h before testing to allow for maximum *in vitro* synthesis of protoporphyrin. However, we found no significant difference in the accuracy of the EPP technique for predicting lead exposure of canvasbacks between blood samples that were held for 24 h and those held for 48 h. Because of potential variation between hematofluorometers, the effect of storage time and the selection of threshold EPP values separating positive and negative populations should be verified with each instrument. However, we have no reason to suspect that blood of other birds with similar rates of lead exposure would react differently.

Screening birds for protoporphyrin concentrations, using hematofluorometry, is a simple, rapid, and inexpensive technique. However, factors that affect the accuracy of hematofluorometry for predicting exposure rates include the precision of the lead analytical technique and the true rate of lead exposure. Analytical precision is of the most significance when a high proportion of the blood samples have lead concentrations near the 0.2 ppm level, providing more opportunities for errors in lead determination. Less than one-third of the canvasback samples we collected had blood lead values between 0.10 ppm and 0.75 ppm wet weight (Figure 1) and our 95% confidence intervals for values around 0.2 ppm were 6 to 12%, suggesting that the error contributed by analytical precision was probably relatively low.

Insofar as the true rate of lead exposure affects the accuracy of hematofluorometry, we found that in a population where 60% of the blood samples had lead concentrations of ≥ 0.2 ppm wet weight, the protoporphyrin technique resulted in a false negative error rate (or underestimation of lead exposure) of 29% in samples held for 24 h before analysis. The false negatives we observed were probably due largely to the fact that, in ducks, the increase in blood protoporphyrin concentrations lags behind that of blood lead concentrations following lead shot exposure. In lead shot dosing studies, peak protoporphyrin concentrations lagged behind peak blood lead concentrations by three to six days (Roscoe et al., 1979; Franson et al., 1986). One may expect a lower rate of false negatives, in populations with lower prevalences of lead exposure, simply because the proportion of birds with elevated blood lead will be lower. The frequency of false positives in our study was less than 10%, indicating that when EPP is used to select blood samples for lead analysis from populations with high rates of exposure, one can expect a high probability that blood lead concentrations will indeed be elevated.

Acknowledgements

We thank H. A. Bateman, Louisiana Department of Wildlife and Fisheries (LDWF), and E. T, Sipco, Catahoula National Wildlife Refuge (CNWR), U.S. Fish and Wildlife Service, for supporting our research. Housing and laboratory facilities were provided by the LDWF and staffs of the CNWR and Dewy Mills State Wildlife Management Area provided logistical support. We thank K. Barrish and D. Finley for assistance with blood collection and analysis. M. Samuel and D. Xiang provided statistical consultation. D. Hoffman and L. Sileo provided helpful comments on the manuscript.

References

Anderson, W. L.: 1992, 'Legislation and Lawsuits in the United States and their Effects on Nontoxic Shot Regulations', in Pain, D. J. (ed.), *Lead Poisoning in Waterfowl.* IWRB Special Publication No. 16. The Internationall Waterfowl and Wetlands Research Bureau, Slimbridge, Gloucester, United Kingdom, pp. 56–60.

Bellrose, F. C.: 1959, 'Lead Poisoning as a Mortality Factor in Waterfowl Populations', *Ill. Nat. Hist. Bull.* **27**, 235–288.

Beyer, W. N., Pattee, O. H., Sileo, L., Hoffman, D. J. and Mulhern, B. M.: 1985, 'Metal Contamination in Wildlife Living Near Two Zinc Smelters', *Environ. Pollut. (Ser A)* **38**, 63–86.

Beyer, W. N., Spann, J. W., Sileo, L. and Franson, J. C.: 1988, 'Lead Poisoning in Six Captive Avian Species', *Arch. Environ. Contam. Toxicol.* **17**, 121–130.

Birkhead, M.: 1982, 'Causes of Mortality in the Mute Swan *Cygnus olor* on the River Thames', *J. Zool. Soc. Lond.* **198**, 15–25.

Blus, L. J., Henny, C. J., Hoffman, D. J. and Grove, R. A.: 1991, 'Lead Toxicosis in Tundra Swans Near a Mining and Smelting Complex in Northern Idaho', *Arch. Environ. Contam. Toxicol.* **21**, 549–555.

Bull, K. R., Every, W. J., Freestone, P., Hall, J. R., Orborn, D., Cooke, A. S. and Stowe, T.: 1983, 'Alkyl Lead Pollution and Bird Mortalities on the Mersey Estuary, UK, 1979–1981', *Environ. Pollut. (Ser A)* **31**, 239–259.

Chupp, N. R. and Dalke, P. D.: 1964, 'Waterfowl Mortality in the Coeur D'Alene River Valley, Idaho', *J. Wildl. Manage.* **28**, 692–702.

DeStefano, S., Brand, C. J., Rusch, D. H., Finley, D. L. and Gillespie, M. M.: 1991, 'Lead Exposure of Canada Geese of the Eastern Prairie Population', *Wild. Soc. Bull.* **19**, 23–32.

Dieter, M. P., Perry, M. C. and Mulhern, B. M.: 1976, 'Lead and PCBs in Canvasback Ducks: Relationship Between Enzyme Levels and Residues in Blood', *Arch. Environ. Contam. Toxicol.* **5**, 1–13.

Franson, J. C., Haramis, G. M., Perry, M. C. and Moore, J. F.: 1986, 'Blood Protoporphyrin for Detecting Lead Exposure in Canvasbacks', in Feierabend, J. S. and Russell, A. B. (eds.), *Lead Poisoning in Wild Waterfowl – A Workshop*, National Wildlife Federation, Washington, DC, pp. 32–37.

Friend, M.: 1985, 'Interpretation of Criteria Commonly Used to Determine Lead Poisoning Problem Areas', *U. S. Fish Wildl. Serv. Fish Wildl. Leafl.* **2**, pp. 4.

Grue, C. E., Hoffman, D. J., Beyer, W. N. and Franson, L. P.: 1986, 'Lead Concentrations and Reproductive Success in European Starlings *Sternus vulgaris* Nesting Within Highway Roadside Verges', *Emviron. Pollut. (Ser A)* **42**, 157–182.

Henny, C. J., Blus, L. J., Hoffman, D. J. and Grove, R. A.: 1994, 'Lead in Hawks, Falcons and Owls Downstream from a Mining Site on the Coeur D'Alene River, Idaho', *Environ. Monit. Assess.* **29**, 267–288.

Hoffman, D. J., Franson, J. C., Pattee, O. H., Bunck, C. M. and Murray, H. C.: 1985, 'Biochemical and Hematological Effects of Lead Ingestion in Nestling American Kestrels *(Falco sparverius)'*, *Comp. Biochem. Physiol.* **80C**, 431–439.

Hohman, W. L., Pritchert, R. D., Pace, R. M., Woolington, D. W. and Helm, R.: 1990, 'Influence of Ingested Lead on Body Mass of Wintering Canvasbacks', *J. Wildl. Manage.* **54**, 211–215.

Hohman, W. L., Moore, J. L. and Franson, J. C.: 1995, 'Winter Survival of Immature Canvasbacks in Inland Louisiana', *J. Wildl. Manage.* **59**, 384–392.

Hutton, M.: 1980, 'Metal Contamination of Feral Pigeons *Columba livia* from the London Area: Part 2-Biological Effects of Lead Exposure', *Environ. Pollut. (Ser A)* **22**, 281–293.

Lamola, A. A., Joselow, M. and Yamane, T.: 1975, 'Zinc Protoporphyrin (ZPP): A Simple, Sensitive, Fluorometric Screening Test for Lead Poisoning', *Clin. Chem.* **21**, 93–97.

Locke, L. N., Kerr, S. M. and Zoromski, D.: 1982, 'Lead Poisoning in Common Loons (*Gavia immer*)', *Avian Dis* **26**. 392–396.

Locke, L. N. and Friend, M.: 1992, 'Lead Poisoning of Avian Species Other Than Waterfowl', in Pain, D. J. (ed.), *Lead Poisoning in Waterfowl*, IWRB Special Publication No. 16. The International Waterfowl and Wetlands Research Bureau, Slimbridge, Gloucester Kingdom, pp. 19–22.

MacDonald, J. W., Randall, C. J., Ross, H. M., Moon, G. M. and Ruthven, A. D.: 1983, 'Lead Poisoning in Captive Birds of Prey', *Vet. Rec.* **113**, 65–66.

Mauser, D. M., Rocke, T. E., Mensik, J. G. and Brand, C. J.: 1990, 'Blood Lead Concentrations in Mallards from Delevan and Colusa National Wildlife Refuges', *Calif. Fish and Game* **76**, 132–136.

Mushak, P.: 1992, 'The Monitoring of Human Lead Exposure', in Needleman, H. L. (ed.), *Human Lead Exposure*, CRC Press, Inc., Boca Raton, FL, pp. 45–64.

Piomelli, S.: 1987, 'The Diagnostic Utility of Measurements of Erythrocyte Porphyrins', *Hematol. / Oncol. Clinics N. Amer.* **1**, 419–430.

Pokras, M. A. and Chafel, R.: 1992, 'Lead Toxicosis From Ingested Fishing Sinkers in Adult Common Loons (*Gavia immer*) in New England', *J. Zoo Wildl. Med.* **23**, 92–97.

Roscoe, D. E., Nielsen, S. W., Lamola, A. A. and Zuckerman, D.: 1979, 'Simple, Quantitative Test for Erythrocytic Protoporphyrin in Lead-Poisoned Ducks', *J. Wildl. Dis.* **15**, 127–136.

Sanderson, G. C. and Bellrose, F. C.: 1986, 'A Review of the Problem of Lead Poisoning in Waterfowl', *Ill. Nat. Hist. Surv. Spec. Publ.* **4**, pp. 34.

SAS/STAT User's Guide: 1990, 'The DISCRIM Procedure', in: *SAS/STAT User's Guide*, Version 6, 4th edn. Vol 1, SAS Institute, Cary, NC, pp. 677–772.

Sileo, L. and Fefer, S. I.: 1987, 'Paint Chip Poisoning of Laysan Albatross at Midway Atoll', *J. Wildl. Dis.* **23**, 432–437.

Taylor, J. K.: 1987, *Quality Assurance of Chemical measurements*. Lewis Publishers, Inc., Chelsea, MI, pp. 210–214.

U. S. Department of the Interior: 1986, *Final Supplemental Impact Statement on the use of Lead Shot for Hunting Migratory Birds in the United States, Fes 86–16*, U.S. Fish Wildl. Serv., Washington, DC, pp. 535.

Wills, D. W. and Glasgow, L. L.: 1964, 'Lead Shot on Catahoula Lake and its Management Implications', *Proc. Ann. Conf. Southeast. Assoc. Fish Wildl. Agencies* **18**, 90–105.

Zar. J. H.: 1984, *Biostatistical Analysis*, 2nd edn., Prentice-Hall, Inc., Englewood Cliffs, NJ, pp. 156–158.